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(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: VAN DER LEE, Frederique, Marianne [NL/NL]; Zeneca Mogen, P.O. Box 628, NL–2300 AP Leiden (NL). SIJMONS, Peter, Christiaan [NL/NL]; Valeriusstraat 210 IV, NL–1075 GK Amsterdam (NL). HETHERINGTON, Alistair, Maculloch [GB/GB]; Lancaster University, Dept. of Biological Sciences, Bailrigg, Lancaster LA1 4YN (GB). HOLROYD, Geoffrey, Heys [GB/GB]; Lancaster University, Dept. of Biological Sciences, Bailrigg, Lancaster LA1 4YN (GB). GRAY, Julie, Elizabeth [GB/GB]; University of Sheffield, Dept. of Molecular Biology & Biotechnology, P.O. Box 594, Sheffiedl S10 1UH (GB).

(74) Agents: GAAL, Jozsef, Christopher et al.; Zeneca Agrochemicals, Intellectual Property Dept., P.O. Box 3538, Jealott's Hill Research Station, Bracknell RG42 6YA (GB).

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(54) Title: POLYNUCLEOTIDE SEQUENCES AND THEIR USE IN A METHOD OF PRODUCING PLANTS WITH AN INCREASED NUMBER OF STOMATA

(57) **Abstract**

A method of producing plants with an increased number of stomata relative to control plants comprises the steps of: (i) inhibiting in plant material the production of fatty acids which stimulate the synthesis of the 14–3–3 class of transcription factors, or otherwise preventing the fatty acids from stimulating the synthesis of the said factors; (ii) selecting the thus inhibited material; and (iii) regenerating the thus selected material into plants. The inhibition may be achieved by sense co–suppression or anti–sense inhibition of an endogenous gene comprising a sequence which is complementary to one which when incubated at a temperature of between 60 and 65 °C in 0.3 strength citrate buffered saline containing 0.1 % SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1 % SDS still hybridises with the sequence depicted in SEQ ID No.1. Preferred sequences for use in this method are depicted as SEQ ID No.1 and 2.

POLYNUCLEOTIDE SEQUENCES AND THEIR USE IN A METHOD OF PRODUCING PLANTS WITH AN INCREASED NUMBER OF STOMATA

The present invention relates, *inter alia*, to genetically engineered plants. In particular the invention relates to polynucleotide sequences and their use in a method of producing plants which possess an increased number of stomata relative to non-transformed (prior art) like plants. Under appropriate conditions the plants of the invention may exhibit increased yields in comparison with the prior art plants. The invention also provides novel regulatory sequences.

In the United Kingdom many commercial growers use natural gas, kerosene burners or liquid carbon dioxide to increase the level of carbon dioxide in their glasshouses. The benefits to glasshouse crops include increases in quality, yield and maximising growth in low external winter light. However, during longer term cultivation the full potential benefits in terms of yield are not achieved by this procedure. A number of physiological and biochemical factors including photosynthetic acclimatisation are believed to contribute to this phenomenon. One important factor however, which has received comparatively little attention is the effect of elevated carbon dioxide on stomatal guard cells. Not only does the stomatal pore reduce in diameter but carbon dioxide also influences guard cell development and specifically reduces the number of stomata which develop on leaves grown at elevated carbon dioxide concentrations. These factors will be expected to result in increases in diffusive resistance and reduced uptake of carbon dioxide. In the case of glasshouse grown lettuce, eggplant, sweet-pepper, cucumber and tomato this is indeed the case. In turn a reduction in the uptake of carbon dioxide would be expected to manifest itself as a reduction in yield.

Another equally important problem for glasshouse growers is crop damage caused by the high levels of humidity which commonly occur as a consequence of the use of modern energy saving practices and or the maintenance of an elevated carbon dioxide environment. To illustrate the seriousness of the problem, it has been estimated that tomato crops grown at high humidity commonly have up to a 10-15% reduction in fruit yield and under sustained conditions of high humidity a reduction in yield of 35% has been recorded. In cucumbers and long season tomatoes cultivated at high humidity, transpiration is suppressed thereby reducing the uptake of nutrients (in particular calcium) causing leaf-scorch symptoms and

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reduced effective and actual leaf areas. In the case of calcium deficiency the associated tissue necrosis makes the crop particularly susceptible to pathogen attack resulting in further decreases in crop potential. In tomatoes cultivation at high humidity results in lower fruit yield and quality. The higher than average humidity also provides an environment which is conducive to breeding for certain pests which can affect the quality of the crop. It would therefore be advantageous to control these pests as well as the deleterious effects resultant from the increased carbon dioxide levels. Of course, due to the concomitant increases in stomatal resistance associated with growth at elevated carbon dioxide, all of the above mentioned problems are likely to be exacerbated.

The present invention alleviates the aforesaid problems by providing plants which specifically respond to elevated carbon dioxide concentrations by increasing the number of stomata on their leaf surfaces. In the absence of other limiting factors this would be expected to increase carbon dioxide uptake for photosynthesis and present a greater effective surface area for water loss resulting in increased transpiration and thereby counteracting calcium deficiency. Data from carbon isotope discrimination studies in wheat, barley, rice and *Phaseolus vulgaris* indicate that genotypes with lower stomatal resistance are higher yielding.

This invention also relates to polynucleotide sequences and variants thereof which are capable of regulating gene expression, particularly in the stomatal guard cells.

The expression of genes in plants is controlled by a number of regulatory components, including nucleic acid and protein elements. Where the plant gene exists as double stranded DNA, the primary steps of expression involve the production of a messenger RNA by a polymerase enzyme. The initiation of this part of the expression process is controlled by a region commonly referred to as the "promoter". The promoter lies upstream (5') of the protein encoding region and may be constitutive or tissue-specific, developmentally-regulated and/or inducible.

Within the promoter region there are several domains which are necessary for full function of the promoter. The first of these domains lies immediately upstream of the structural gene and forms the "core promoter region" containing consensus sequences, normally 70 base pairs immediately upstream of the gene. The core promoter region contains the characteristic CAAT and TATA boxes plus surrounding sequences, and represents a transcription initiation sequence which defines the transcription start point for the structural

gene. The precise length of the core promoter region is indefinite but it is usually easily recognisable. Such a region is normally present, with some variation, in all promoters. The base sequences lying between the various well-characterised "boxes" appear to be of lesser importance.

The presence of the core promoter region defines a sequence as being a promoter: if the region is absent, the promoter is non-functional. Furthermore, the core promoter region is insufficient to provide full promoter activity. A series of regulatory sequences, usually upstream of the core, constitute the remainder of the promoter. The regulatory sequences determine expression level, the spatial and temporal pattern of expression and, for an important subset of promoters, expression under inductive conditions (regulation by external factors such as light, temperature, chemicals, hormones).

Manipulation of crop plants to alter and/or improve phenotypic characteristics (such as productivity quality or yield) may require the expression of heterologous genes in plant tissues. Such genetic manipulation therefore relies on the availability of means to drive and to control gene expression as required; for example, on the availability and use of suitable promoters which are effective in plants and which regulate gene expression so as to give the desired effect(s) in the transgenic plant. It is advantageous to have the choice of a variety of different promoters so that the most suitable promoter may be selected for a particular gene, construct, cell, tissue, plant or environment.

Promoters (and other regulatory components) from bacteria, viruses, fungi and plants have been used to control gene expression in plant cells. Numerous plant transformation experiments using DNA constructs comprising various promoter sequences fused to various foreign genes (for example, bacterial marker genes) have led to the identification of useful promoter sequences. It has been demonstrated that sequences up to 500-1000 bases in most instances are sufficient to allow for the regulated expression of foreign genes. However, it has also been shown that sequences much longer than 1 kb may have useful features which permit high levels of gene expression in transgenic plants. A range of naturally-occurring promoters are known to be operative in plants and have been used to drive the expression of heterologous (both foreign and endogenous) genes in plants: for example, the constitutive 35S cauliflower mosaic virus promoter, the ripening-enhanced tomato polygalacturonase promoter (Bird et al, 1988, Plant Molecular Biology, 11:651-662), the E8 promoter (Diekman & Fischer, 1988, EMBO, 7:3315-3320) and the fruit specific 2A11 promoter (Pear et al, 1989, Plant Molecular Biology, 13:639-651) and many others.

According to the present invention there is provided a method of producing plants with an increased number of stomata relative to control like plants comprising the steps of: (i) inhibiting in plant material the production of fatty acids which stimulate the synthesis of the 14-3-3 class of transcription factors, or otherwise preventing the fatty acids from stimulating the synthesis of the said factors; (ii) selecting the thus inhibited material; and (iii) regenerating the thus selected material into plants and selecting from the population of regenerants those plants having an increased number of stomata relative to control like plants.

The invention further provides a method of producing plants with an increased number of stomata relative to control like plants comprising the steps of: (i) inhibiting the function, or otherwise disrupting the activity, of an endogenous gene comprising a polynucleotide sequence depicted as SEQ ID No 1 or SEQ ID No 2 or SEQ ID No 8.

(ii) selecting the thus inhibited material;

(iii) regenerating the thus selected material into plants and selecting from the population of regenerants those plants having an increased number of stomata relative to control like plants.

In performing the method of the present invention as described above, the endogenous gene may comprise a polynucleotide sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1 or SEQ ID No 2 or SEQ ID No 8.

Also provided is a method of producing plants with an increased number of stomata relative to control like plants comprising the steps of: (i) transforming plant material with a polynucleotide comprising the sequence depicted as SEQ ID No 1 or 2 or SEQ ID No 8.

(ii) selecting the thus transformed material;

(iii) regenerating the thus selected material into plants and selecting from the population of regenerants those plants having an increased number of stomata relative to control like plants.

The polynucleotide used in the above mentioned method may comprise one which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same

temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No 1 or SEQ ID No 2 or SEQ ID No 8. It is particularly preferred that the polynucleotide used in the method is in antisense orientation. Plants with an increased number of stomata may be selected on the basis of a difference between non transformed control plants and the thus transformed plants when both are subjected post germination to at least one of the following: (i) elevated carbon dioxide concentration; (ii) elevated calcium; (iii) extremes of temperature or pressure; (iv) reduced water availability; (v) elevated environmental pollutant gases, such as ozone, oxides of nitrogen or sulphur, and (vi) elevated light conditions. Preferably the carbon dioxide concentration is greater than about 450 parts per million (ppm). More preferably the carbon dioxide concentration is greater than about 550 parts per million. More preferably the carbon dioxide concentration is greater than about 650 parts per million.

The present invention also provides morphologically normal fertile whole plants and the seed and progeny thereof regenerated from the material described above and having an increased number of stomata relative to a control like plants. Plants transformed according to the methods of the present invention may include; soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, grape, vines, cucumbers, peppers, citrus and nut plants.

The present invention additionally provides the use of a polynucleotide comprising the sequence depicted as SEQ ID No 1 or SEQ ID No 2 or SEQ ID No 8 in a method of producing plants with an increased number of stomata relative to control like plants. Other polynucleotides which can be used in this method may comprise a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1 or SEQ ID No 2 or SEQ ID No 8. The polynucleotides for use in this method may be under expression control of a plant operable promoter and may further comprise a transcription termination region which is downstream of the protein encoding region of the said polynucleotide. In particular the following promoters may be used: CaMV35S; FMV35S; NOS; OCS and E9. More preferably the promoter may be a stomatal guard cell specific promoter. Even more preferably the promoter may be comprised by the polynucleotide sequence depicted as SEQ ID No 2 or SEQ ID No 8.

Also provided is an isolated polynucleotide comprising the sequence depicted as SEQ ID No 2 or SEQ ID No 8..

The present invention still further provides an expression regulatory sequence comprising the sequence depicted as SEQ ID No 2 or SEQ ID No 8. Surprisingly, it has been found that this sequence is capable of providing for expression of heterologous genes in the stomatal guard cell. The regulatory sequences provided in the present invention can be used in combination with the polynucleotides and methods described above. The person skilled in the art will however, recognise that these regulatory sequences can be used in combination with any other polynucleotide in any method where transcription is particularly required in the stomatal guard cell.

According to the present invention there is further provided a polynucleotide which comprises a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1, but does not so hybridise when the said temperature is between 65 and 70°C. The polynucleotide may be used to produce plants which increase their stomatal index (as described below) rather than decrease it when subjected to conditions of elevated carbon dioxide concentration.

The polynucleotide may comprise the sequence depicted in SEQ ID No. 2 or SEQ ID No 8. The protein encoding region comprised by the polynucleotide may be bounded by a plant operable promoter and terminator. Such promoters and terminators, which are *per se* not germane to the invention, are well known to the skilled man and include, for example, the CaMV35S, FMV35S, NOS, OCS and E9 (derived from the small subunit of RUBISCO) promoters and terminators. It is particularly preferred, however, that the protein encoding region of the polynucleotide according to the invention is under expression control of a stomatal guard cell specific promoter. The skilled man understands that the term "specific" does not necessarily mean "solely restricted to" so that expression of the said sequence cannot be found anywhere else within the plant regenerated from material transformed so as to comprise such a region.

The invention also includes a plant transformation vector comprising the present inventive polynucleotide.

Within the vector the protein encoding region (or a substantial part of it) may be in an anti-sense orientation when compared with that depicted in SEQ ID Nos. 1, 2 and 8, so that

the RNA product of the region is capable of causing - in plant material comprising it - suppression of endogenous genes with which the protein encoding region exhibits substantial identity. By "substantial" is meant at least 70% identical when related to sequence.

The invention further provides the translational product encoded by the polynucleotide of the invention, particularly in the case that it has the activity of a fatty acid elongase.

The invention still further provides plant material which has been transformed with the polynucleotide or vector of the invention, or a polynucleotide comprising a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1.

The plant material may have been, or may subsequently be further transformed with a polynucleotide comprising a region encoding a protein capable of providing the plant with resistance or tolerance to herbicides, insects, desiccation and/or fungal, bacterial or viral infections.

The protein capable of providing for herbicide resistance may be selected from the group consisting of glyphosate oxido-reductase (GOX), 5-enol-pyruvyl-3-phosphoshikimate synthetase (EPSPS), phosphinothricin acetyl transferase (PAT), hydroxyphenyl pyruvate dioxygenase (HPPD), glutathione S transferase (GST), cytochrome P450, Acetyl-COA carboxylase (ACCase), Acetolactate synthase (ALS), protoporphyrinogen oxidase (PROTOX), dihydropteroate synthase, polyamine transport proteins, superoxide dismutase (SOD), bromoxynil nitrilase, phytoene desaturase (PDS), the product of the *tfd*A gene obtainable from *Alcaligenes eutrophus*, and known mutagenised or otherwise modified variants of the said proteins.

The polynucleotide with which the plant material may be transformed may comprise 5' of the protein encoding regions which encode: (i) a peptide which is capable of targeting the translation products of the regions to plastids such as chloroplasts, mitochondria, other organelles or plant cell walls; and/or (ii) non-translated translational enhancing sequences.

The polynucleotide may be codon-optimised, or otherwise altered to enhance at least transcription once it is incorporated into plant material. Thus the polynucleotide used to transform the material may be modified in that mRNA instability encoding motifs and/or fortuitous splice regions may be removed, or plant preferred codons may be used so that

expression of the thus modified polynucleotide in a plant yields substantially similar protein having a substantially similar activity/function to that obtained by expression of the unmodified polynucleotide in the organism in which the protein encoding regions of the unmodified polynucleotide are endogenous, with the *proviso* that if - in respect of the herbicide resistance conferring regions - the thus modified polynucleotide comprises plant preferred codons, the degree of identity between the protein encoding regions within the modified polynucleotide and like protein encoding regions endogenously contained within the said plant and encoding substantially the same protein is less than about 70%.

Transformation techniques are well known and include particle mediated biolistic transformation, *Agrobacterium*-mediated transformation, protoplast transformation (optionally in the presence of polyethylene glycols); sonication of plant tissues, cells or protoplasts in a medium comprising the polynucleotide or vector; micro-insertion of the polynucleotide or vector into totipotent plant material (optionally employing the known silicon carbide "whiskers" technique), electroporation and the like.

The invention still further provides a morphologically normal fertile whole plant regenerated from the material mentioned in the paragraph immediately preceding the last and the progeny of such plants, the seed of such plants and progeny, and parts of such plants and progeny. The transformed inventive plants include small grain cereals, oil seed crops, fibre plants, fruit, vegetables, plantation crops and trees. Particularly preferred such plants include soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, grape, cucumbers, peppers, citrus and nut plants. Particularly preferred parts include cut flowers.

The invention still further provides a method of producing plants with an increased number of stomata relative to control plants comprising the steps of:

(i) inhibiting in plant material the production of fatty acids which stimulate the synthesis of the 14-3-3 class of transcription factors, or otherwise preventing the fatty acids from stimulating the synthesis of the said factors;

(ii) selecting the thus inhibited material; and

(iii) regenerating the thus selected material into plants.

In a preferred embodiment of the method the production of the fatty acids, which stimulate or otherwise enhance the synthesis of the transcription factors, is inhibited by either sense cosuppression of an endogenous plant gene encoding a protein involved in the

biosynthetic pathway of the fatty acids, or else by anti-sense inhibition of the expression of the same gene. Anti-sense inhibition techniques are well know, developed and used routinely by persons skilled in the art. Usually, the inhibition technique is effected in the plant through the production of an antisense mRNA which is complementary to and capable of hybridising with the sense mRNA produced by the endogenous gene.

The method of the present invention may comprise the steps of:

(i) inhibiting the function, or otherwise disrupting the activity, of an endogenous plant gene comprising a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1;

(ii) selecting the thus inhibited material;

(iii) regenerating the thus selected material into plants.

The inventive plants typically contain at least 10% more stomata than do prior art like plants. It is preferred that they contain at least 15% more stomata, more preferred that they contain at least 20% more stomata, still more preferred that they contain at least 25% more stomata, and yet still more preferred that they contain at least 30% more stomata.

The person skilled in the art will appreciate that there are numerous ways of inhibiting or otherwise disrupting the function of a gene. Whilst sense co-suppression and anti-sense inhibition are particularly preferred techniques, it is also possible to use the so called chimeroplasty technique of *in situ* mutagenesis of an endogenous gene. The technique *per se* is not germane to the present invention but, briefly, it involves the introduction into plant material of mixed ribo-deoxyribonucleic acids which comprise a region (typically less that 100 nucleotides in length) which is complementary to a target sequence in the endogenous gene, with the *proviso* that within the region of complementarity there is a "mismatch" which becomes represented in the endogenous gene *via* the action of DNA repair and replication enzymes. The mismatch typically occurs within a region of the gene encoding the active site of an enzyme, the activity of which is consequentially abolished or at least severely curtailed. In summary then, once the endogenous gene to be silenced has been identified, any gene suppression technique can be applied. Additionally the person skilled in the art is also free to use techniques available within the art to enhance the efficacy of suppression of the desired gene. On such method involves the use of an inverted repeat

sequence and is described in the International Application, PCT publication number WO98/53083 which is incorporated herein by reference.

In performing the method of the invention the skilled man may, however, prefer to transform plant material with a polynucleotide comprising a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1. Alternatively, the plant material may be transformed with a polynucleotide which comprises a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1, but does not so hybridise when the said temperature is between 65 and 70°C. A particularly preferred polynucleotide for use in this method comprises the sequence depicted in SEQ ID No. 2 or in SEQ ID No 8.

The plants with an increased number of stomata may be selected on the basis of a difference between non transformed control plants and the thus transformed plants when both are subjected post germination to at least one of the following: (i) elevated carbon dioxide concentrations, (ii) elevated calcium; (iii) extremes of temperature or pressure; (iv) reduced water availability; (v) elevated environmental pollutant gases, such as ozone, oxides of nitrogen or sulphur, for example; (vi) elevated light conditions. The said difference may be selected from the group consisting of: (i) delayed flowering; (ii) altered growth characteristics; and (iii) an elevated stomatal index. Alternatively, the plants may be selected on the basis of resistance to an antibiotic, which resistance is produced by an antibiotic resistance conferring gene which has been co-introduced into the plant material together with genes capable of increasing the number of stomata. The person skilled in the art is aware of the term "stomatal" index, which in essence is the number of stomata in a particular area of a leave (for example) when expressed as a percentage of the total number of cells contained within that area. Stomatal index may be defined as follows:

SI= stomatal frequency/ epidermal cell frequency + stomatal frequency X 100.

The invention also includes plants - and their progeny - which eventually result from the crossing of plants of the invention with control non transformed sexually compatible plants. The plants or progeny may be homozygous for the transgene.

The invention still further includes plants which result from the method disclosed above, the progeny of such plants, the seed of such plants and progeny, and parts of such plants and progeny. Particularly preferred parts are fruits, flowers and seeds.

The invention still further includes the use of the polynucleotide or vector of the invention in a method for the production of plants which have an increased number of stomata relative to non transformed control plants.

The invention will be further apparent from the following description, taken together with the associated Figures and Sequence Listings.

SEQ ID No. 1 shows the sequence of a fatty acid elongase gene designated FAE-1 which is expressed in seeds. SEQ ID No. 1 can be found on page 316 of The Plant Cell, Vol 7, 1995.

SEQ ID No. 2 shows the sequence of a gene which may be related to - but which is not obviously derivable from - the sequence depicted in SEQ ID No. 1.

SEQ ID Nos. 3 and 4 depict PCR primers which were used in the provision of the vectors pFL30 and pFL44.

SEQ ID Nos 5, 6 depict GUS gene primers.

SEQ ID No 7 depicts a primer for use with pfu polymerase.

SEQ ID No 8 shows the sequence of a gene which may be related to - but which is not obviously derivable from - the sequence depicted in SEQ ID No. 2.

Figure 1 depicts schematically the proof reading PCR DNA clone of *Arabidopsis* C24, line 590, which is cloned into the pGEMT vector (Promega).

Figure 2 depicts the vector pMOG553.

Figure 3 depicts schematicall the relationship between the clones pFL44 and pFL30.

Figure 4 depicts the pFL13 scematic drawing.

Figure 5 depicts the cloning scheme of pMOG1017.

Figure 6 depicts Arabidopsis leaves transformed with the construct pMOG1017 following histochemical staining for GUS activity in the guard cells. In this figure, the "black spots" represent the staining which is naturally blue in colour.

Figure 7 is a magnification of the leaf shown in figure 6 more clearly showing that the stianing is present in the stomata, more specifically in the guard cells.

Examples

This Example illustrates the provision of a gene involved in the control of stomatal number. The gene was initially isolated through a promoter trap screen in *Arabidopsis* ecotype C24. The trapping construct, pMOG553, consisted of the transcribed sequence of the ß Glucuronidase enzyme synthesis gene (GUS) linked to the Hygromycin B phosphotransferase gene (*hyg*) both from *E-coli*. Expression of this construct through an endogenous 'trapped' promoter allowed localisation of gene expression through staining for GUS activity and an antibiotic selection marker for isolation of mutants. On the basis of the GUS reporter gene a primary mutant (M1) plant (it and the progeny thereof hereinafter being designated Tag 590) exhibiting guard cell specific expression was identified, indicating that a guard cell specific gene had been disrupted.

Provision of Tag 590 plants

Goddijn *et al.* (The Plant J. 4 (5), 863-873, 1993) describes the production of the tagging construct pMOG553 (promoterless GUS on T-DNA). pMOG553 is depicted schematically in Figure 2. This reference also describes the transformation of *Arabidopsis thaliana* with this construct and provides an account of the histochemical assay used for GUS analysis. Concerning the GUS analysis, whole leaves (including cotyledons) or leaf sections were assayed for GUS activity by submerging them in assay medium (0.1M phosphate buffer pH7, 10mM EDTA, 0.5mM potassium ferricyanide, 0.5mM potassium ferrocyanide, 2.0mM X-Gluc (Gold Biotechnology), 0.1% Triton X-100) (Stomp, 1992). Large tissue samples were assayed in microfuge tubes while small seedlings or cotyledons were placed in microtitre plate wells. Following 15 minutes continuous vacuum infiltration, samples were incubated for 22 hours at 37°C. Tissue clearing, to remove chlorophyll and oxidised phenolic compounds, was achieved by mounting the slides or submerging the samples in chloralactophenol (CLP)(2:1:1 mix of chloral hydrate, lactic acid and phenol crystals (Sigma), Beckman and Engler, 1994). Results were recorded photographically on Fujichrome Sensia 200 slide film.

Temporal and spatial expression of the GUS gene

GUS expression events in the Tag 590 line were predominantly localised to stomatal guard cells (or hydatodes) suggesting that a guard cell specific gene had been disrupted. This tagged line provides an opportunity to isolate a specific promoter that would enables transgenes to be expressed exclusively in guard cells. *Inter alia*, it is thus possible to exploit the guard cell as an *in vivo* model for the analysis of the role of putative signal transduction gene products in plants and enables the manipulation of stomatal cell gene expression to engineer stomatal cell behaviour.

A total of 12 Tag 590 S2 lines were identified on the basis of selection on hygromycin (lines 5/1 - 5/12). Line 5 was then used to confirm the GUS expression results mentioned above. On the basis of the intensity of histochemical staining, expression was maximal in the first two true leaves of two week old plants. GUS expression was localised to the stomatal guard cells. In older leaves GUS staining was much less intense and required incubation overnight in the substrate for detection. This result may indicate that expression is maximal in the guard cells of young developing plants and that the gene is weakly expressed in mature plants. Alternatively, it may reflect the fact that in mature plants the GUS protein is less stable or that the guard cells are less accessible to the substrate. In the older leaves staining was also observed in the hydrathodes and vascular tissue. Subsequent experiments on all 12 lines showed lines 3, 9 and 11 to had the highest levels of GUS expression.

Isolation of the disrupted gene in Tag 590 plants.

Genomic DNA (gDNA) was isolated from Tag 590 plant material (rosette leaves). The copy No. of the T-DNA insert was determined by restriction enzyme analysis of the said genomic DNA using EcoR V, Msc I with the 5' part of the GUS gene being used as a probe. This restriction regime yielded a single band on suitable electrophoresis. An inverse PCR was performed on the gDNA of Tag590 using the following GUS gene primers (according to the method described by Barthels, see p. 2131, Fig. 7 of that document).

primer 7: 5'-GTA.ATG.CTC.TAC.ACC.ACG.CCG-3' (SEQ ID No. 5).
primer5: 5'-CTT.TCC.CAC.CAA.CGC.TGA.TC-3' (SEQ ID No. 6).

For the EcoR V fragment a band of about 1.8 kb was obtained from which the tag had a size of 1.6 kb. An identical result was obtained for the Msc I fragment. Cloning of this fragment

in the pGEM T vector from PROMEGA resulted in a plasmid designated pFL13, see Figure 4. This clone was partially sequenced and a Pst1 fragment of 1.4 kb was isolated and used as a probe (see Fig. 4) to screen a genomic DNA library of wild type C24 *Arabidopsis* constructed by the method described in Barthels *et al.* 1997 Plant Cell, Vol 9, pp 2119-2134. After 3 rounds of colony purification, five clones were isolated, two of which were identical and three of which were identical. A 5.5 Kb fragment was cloned into the BglII site of pUC28, (which is a pUC18 vector modified to contain extra Bg1II restriction enzyme recognition sites), to yield a plasmid designated as pFL30.

The following primers were used to in the production of a PCR fragment which was generated on genomic DNA from original TAG590 using pfu polymerase (Proofreading capacity). This fragment was then cloned into the pGEMT making the construct labelled as pFL44.

GUS 9: 5'-CAG.AAA.CTT.ACG.TAC.ACT.TTT.C-3' (SEQ ID 7)

590-5' : 5'- CAT.CTT.CTT.CTA.TGC.CTA.CTC -3' (SEQ ID 3)

Whilst pFL44 does not contain any recognizable promoter sequences, it has been shown that it gives specific expression when transformed to plants indicating that it comprises some tissue specific regulatory elements.

The PCR fragment (pFL44) has been completely sequenced on both strands. It appears that the GUS tag has inserted within a gene with extensive similarity but clearly non-identity to an *Arabidopsis* gene that has been previously identified by transposon tagging experiments and is designated as FAE I. The sequence depicted in SEQ ID No. 2 contains at least two exons and does have putative translation initiation and termination points but appears to encode a peptide significantly shorter than FAE I (110 and 12 amino acids missing at the N- and C- terminal ends respectively). Therefore it is possible that there are additional coding regions that are not within this cloned fragment. The GUS tag has inserted 3' to the second exon (180bp 3' to the putative translation stop codon) this is probably close to the end of the transcribed sequence or alternatively maybe within an intron. It is unlikely that another gene could be within this distance from the Fae-like coding sequences.

The corresponding region of the genomic clone (pFL30) has been sequenced using the same Internal primers. This longer clone is sequenced with a view (i) to identifying further exons, if they exist; and (ii) to characterise the potential gene promoter regions which may lie upstream of the identified coding sequence. FAE I is thought to encode a fatty acid

elongase necessary for the production of very long chain fatty acids. A transposon tagged FAE I mutant fails to accumulate fatty acids longer than C18 (i.e. 20:0, 20:1 and 22:1) in its seed. FAE I is thought to encode a seed specific ketoacyl synthase which catalyses the condensation reaction with malonyl CoA. A region of protein having approximately 50 amino acid in FAE I has been identified which shares some sequence similarity to regions within other plant malonyl CoA condensing enzymes (e.g. CHS and STS). At the DNA level there is some similarity to 4 Arabidopsis ESTS, T6700, T44939, T44368 and ATTS1282. These homologies lie entirely within the proposed coding regions of the SEQ ID No. 2 sequence. This implies that, including FAE I, there are at least four other sequences with similarity to the Tag 590 coding region in the *Arabidopsis* genome. It is possible that these six genes have different expression patterns with FAE I being seed specific and Tag 590 guard cell specific. These results thus suggest that the Tag 590 gene encodes a putative fatty acid elongase which is expressed specifically in developing guard cells. This enzyme plays a role in determining stomatal density in response to altered carbon dioxide concentration (described below).

Use of the FAE sequence as a stomatal guard cell promoter

This experiment was designed to illustrate that the FAE sequence can act as a stomatal guard cell specific promoter. The plasmid pFL44 was digested with SnaBI(Located in 5' end of GUS gene) providing a 1.8 kb fragment. This fragment was cloned in the SnaBI/SmaI site of pFL7 (which is a multicopy construct harbouring a promoterless GUS gene and 35S terminator) replacing 5'end of GUS producing a construct labelled the "new multicopy construct". From this "new multicopy construct" a EcoRI/BamHI fragment harbouring the following elements was cloned in pMOG800; a Tag590 "promoter"; GUS gene and 35S terminator. The final construct pMOG1017 was used for the Arabidopsis C24 transformation following which 40 transgenic lines were generated and their leaves were histochemically tested for GUS expression. 14 Lines show clearly specific expression of the GUS gene in the stomata only see photo's 1 or 2. The remaining 26 lines did not show clear expression and were thought to be low or non expressors.

Plants and growth conditions

Twelve guard cell specific GUS expressing M2 lines of the single M1 plant were produced by selfing. The M2 lines have been used for all characterisation analysis. Prior to

sowing, seed was surface sterilised on filter paper (1 wash 10% v/v bleach (5 mins) followed by washes (5x1ml) with sterile distilled water). The seed was germinated on half strength Murashige and Skoog basal media with the addition of 1% w/v sucrose and 0.6% w/v tissue culture grade agar, pH 5.8 (KOH). Standard 10cm culture plates were prepared either without the addition of antibiotics for wild type seed or with 10mg/l Hygromycin B (Calbiochem) for selection of mutant seedlings. Sown plates were subjected to a 48 hour vernalisation (0-4°C) before transfer to growth conditions maintained throughout the lifecycle (22°C day, 17°C night; 10/14 hour day/night regime; R/H >60%; 400w Osram metal halide 250-300 µmol $m^{-2}sec^{-1}$). Following selection plants were transferred to soil (3:1 mix SHL all purpose compost (William Sinclair Horticulture Ltd) : fine 'silver' sand).

Morphological Characterisation

Controlled environment chambers were used to grow plants under ambient (350-450ppm) and elevated (ambient +650ppm) carbon dioxide. Under these conditions flower and leaf appearance, plant development and flowering time were all recorded. Wild type C24 were used as untransformed controls and 35S constitutive GUS expressors were used as GUS positive controls. Plants were transferred to the chambers at 16 or 35 days after exposure to lighting (dal). In the case of the 16 day age group, plants were grown on to 35 days whilst the older plants were grown to seed set before termination of the experiments. Initiation of flowering was recorded for individual plants up to 67 days. On harvest all plants were photographed. Xantopren dental impression material (Dental Links Products) was used to take impressions of the abaxial epidermis (Weyers and Johansen 1985) from 2 to 3 leaves per plant, choice of leaf being based on comparative size rather than leaf number. Optically clear acetone based varnish was used to make positives from the Xantopren impressions. Stomatal and epidermal cell counts per unit area ($9.2^{-2}mm^2$).were taken from three different parts of each positive under light microscopy at x200 magnification. These data were used to calculate average stomatal index (SI) values (SI= stomatal frequency/ epidermal cell frequency + stomatal frequency X 100) for each line under ambient and elevated CO_2. The results indicated that there was no difference in either stomatal frequency or stomatal index between C24 and Tag 590 plants under ambient CO_2 conditions. In marked contrast to the reduction in stomatal index seen in C24 plants under elevated carbon dioxide Tag 590 plants exhibit an increase in stomatal index. This result was seen in both 35 and 67 dal plants.

A histochemical assay for GUS expression was carried out to verify the presence or absence of the mutation TAG 590 in all lines. C24 and 35S/GUS (constitutive Gus expression) plants were included as controls.

The phenotype of the Tag 590 line

On the basis of the GUS expression data a guard cell specific gene has been disrupted. Guard cells are known to exhibit two distinct responses to elevated carbon dioxide (CO_2). One of these, the reduction in stomatal aperture is well known to commercial growers of glasshouse crops where it prevents the full benefits of growth at elevated CO_2 being transferred into yield. The other is a developmental response and is manifested in certain species by a reduction in the number of stomata in plants grown under elevated CO_2. The results described below come from experiments in which the phenotype of the Tag 590 plants and C24 controls were compared under elevated and ambient CO_2.

Experiment 1

Three lines were used in this experiment: (i) a C24 control (see above) (4 plants/treatment); (ii) line 5/10 (7 plants/treatment; this is a line with very low expression of the GUS gene); and (iii) line 5/5 (2 plants /treatment of this GUS expressing line). The plants were 4 weeks old when they were transferred to glasshouses and maintained for 17 days at either ambient or 250 ppm CO_2 above ambient. At the end of the experiment stomatal numbers were assessed.

Results of Experiment 1

The most striking result was an alteration in flowering time in the Tag 590 5/5 line. Compared to either the C24 or the 5/10 line controls the initiation of flowering was noticeably delayed in the 5/5 line. When stomatal numbers were counted (Table 1) it was found that there was no difference between ambient and elevated CO_2 conditions for the C24 or 5/10 plants. However, in marked contrast the number of stomata in the strongly GUS expressing line (5/5) had increased.

Table 1. Effect of growth at ambient and elevated C02 on stomatal number

stomatal index		
	ambient	elevated
C24	28.8	27.2
590 5/10	29.2	29.2

590 5/5	27.6	31.6

Experiment 2

The objective of this experiment was to use more carefully controlled growth conditions to investigate the TAG 590 phenotype more accurately. In this experiment twin, matched, environmentally controlled growth cabinets in which the CO_2 concentration was computer controlled and logged throughout the experiment were used. In the first cabinet an ambient level of CO_2 was maintained for the duration of the experiment. In the second cabinet the CO_2 level was kept at 650 ppm above ambient. This was a higher concentration that used in Experiment 1 but was chosen as it more accurately reflects the CO_2 regimes employed by commercial growers.

Results of Experiment 2

The seeds were germinated in a growth room, potted on and at 45 days old were transferred to growth cabinets. They were then grown for a further 22 days and at the end of the experiment flowering was recorded as was stomatal number. It is important to note that stomatal number was only recorded in leaves which had grown during the experimental treatment. The delayed flowering of the tagged plants in elevated CO_2 was seen again in this experiment. All the tagged lines flowered later than the controls in both elevated and ambient treatments (delayed by approximately 7 days). The results of the stomatal number determination are summarised in Table 2 where it is apparent that stomatal number was reduced in control plants grown in elevated CO_2 but that in both tagged lines it was increased thus confirming the results of the previous experiment which indicated that stomatal numbers increased in response to elevated CO_2.

Table 2. Effect of growth at elevated and ambient CO_2 on stomatal number

	stomatal density	
	ambient	elevated
C24	13.1 (6 plants)	11.3 (6 plants)
512	14.4 (6 plants)	17.1 (3 plants)
Sill	13.9 (6 plants)	17.2 (5 plants)

These results imply that the tagged mutant lines are unable to respond normally to altered CO_2 concentrations. Instead of exhibiting a decrease in stomatal density under high CO_2 concentrations they respond by increasing stomatal density. Thus, it appears that a gene involved in stomatal patterning in response to CO_2 has been disrupted. The sequence of the

putatively disrupted gene has been identified and is given in SEQ ID No. 2. The promoter region of this gene is of great interest since it is supposed that it contains: (i) elements which direct guard cell specific gene expression; and (ii) CO_2 responsive elements. The mutant 590 phenotype and the mutated gene have been characterised. Our results lead us to believe that the gene and or its product are: (i) involved in the control of stomatal density; (ii) are responsive to CO_2 : and (iii) that the gene product is or is related to a fatty acid elongase (FAE) which is involved in the synthesis of long chain fatty acids. Together these results suggest that a long chain fatty acid or its metabolite plays an important role in the control of stomatal patterning and that the non-coding regions of this gene include guard cell-specific and CO_2-inducible promoters. In addition to being of interest to commercial plant biotechnologists wishing to engineer stomatal behaviour, the gene and its promoter may be valuable in the context of glasshouse crops. In this situation, in an attempt to maximise carbon acquisition (and hence yield), commercial growers frequently cultivate their plants at high CO_2 concentrations. However, it is recognised that the potential benefits are reduced by the elevated CO_2 induced reduction in stomatal conductance. An increase in stomatal cell number compensates - at least to some extent - for this effect.

The delay in flowering observed in the tagged mutant lines may result from a secondary effect of the gene disruption. That is, the altered stomatal density in these plants causes changes in the rate of carbon assimilation which in turn affects the initiation of the floral meristem.

The person skilled in the art will recognise that the invention is not limited to that described above, many variations being included with its scope, the matter for which protection is sought being defined by the claims. For example, although the Example discloses that the stomatal cell specific promoter which drives expression of the sequence depicted in SEQ ID No. 2 is derived from *Arabidopsis*, blotting experiments have shown that the promoter sequence is present in plants other than just *Arabidopsis*. Such plants include, for example, potato, tomato, and carrot. Moreover, although not specifically exemplified, transformation of the sequences depicted in SEQ ID Nos. 1 and/or 2 into plant material so as to disrupt the activity of endogenous genes having like sequences can be expected to provide plants which when regenerated from such transformed material, will have an increased number of stomata when subjected to elevated carbon dioxide concentrations when compared with like non transformed control plants. In addition to this there is no reason to doubt that

the sequence represented as SEQ ID No 8 will act in the same way as SEQ ID No 2 with respect to the stomatal specific promoter activity.

References (additional to those specifically mentioned above)

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Lindsey, K., Wei, W., Clarke, M.C., McArdle, H.F., Rooke, L.M., Topping, J.F. (1993) Tagging genomic sequences that direct transgene expression by activation of a promoter trap in plants. Transgenic Res. 2, 33-47.

Stomp, A.M. (1992). Histochemical localisation of *B*-glucuronidase In Gus Protocols: Using the GUS Gene as a Reporter of gene expression (Gallagher, S.R., ed.). San Diego: American Press. pp. 103-113.

Weyers, J.D.B. and Johansen, L.G. (1985) Accurate estimation of stomatal aperture from silicone rubber impressions. New Phyt. 101, 109-115.

CLAIMS

1. A method of producing plants with an increased number of stomata relative to control like plants comprising the steps of:

(i) inhibiting in plant material the production of fatty acids which stimulate the synthesis of the 14-3-3 class of transcription factors, or otherwise preventing the fatty acids from stimulating the synthesis of the said factors;

(ii) selecting the thus inhibited material; and

(iii) regenerating the thus selected material into plants and selecting from the population of regenerants those plants having an increased number of stomata relative to control like plants.

2. A method of producing plants with an increased number of stomata relative to control like plants comprising the steps of:

(i) inhibiting the function, or otherwise disrupting the activity, of an endogenous gene comprising a polynucleotide sequence depicted as SEQ ID No 1 or SEQ ID No 2.

(ii) selecting the thus inhibited material;

(iii) regenerating the thus selected material into plants and selecting from the population of regenerants those plants having an increased number of stomata relative to control like plants.

3. A method according to claim 2 wherein the said endogenous gene comprises a polynucleotide sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1 or SEQ ID No 2.

4. A method of producing plants with an increased number of stomata relative to control like plants comprising the steps of:

(i) transforming plant material with a polynucleotide comprising the sequence depicted as SEQ ID No 1 or 2.

(ii) selecting the thus transformed material;

(iii) regenerating the thus selected material into plants and selecting from the population of regenerants those plants having an increased number of stomata relative to control like plants.

5. A method according to claim 4 wherein the said polynucleotide comprises one which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1 or SEQ ID No 2.

6. A method according to claim 4 or claim 5 wherein the said polynucleotide is in antisense orientation.

7. A method according to any one of claims 1 to 6 wherein the plants with an increased number of stomata are selected on the basis of a difference between non transformed control plants and the thus transformed plants when both are subjected post germination to at least one of the following: (i) elevated carbon dioxide concentration; (ii) elevated calcium; (iii) extremes of temperature or pressure; (iv) reduced water availability; (v) elevated environmental pollutant gases, such as ozone, oxides of nitrogen or sulphur, and (vi) elevated light conditions.

8. A method according to claim 7 wherein the said carbon dioxide concentration is greater than about 450 parts per million.

9. A method according to claim 8 wherein the said carbon dioxide concentration is greater than about 650 parts per million.

10. Morphologically normal fertile whole plants, and the seed and progeny thereof regenerated from the material of any one of claims 1 to 9 which plants and progeny have an increased number of stomata relative to a control like plants and progeny.

11. A morphologically normal fertile whole plant according to claim 10 selected from the group consisting of soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, grape, vines, cucumbers, peppers, citrus and nut plants.

12. Use of a polynucleotide comprising the sequence depicted as SEQ ID No 1 or SEQ ID No 2 in a method of producing plants with an increased number of stomata relative to control like plants.

13. Use according to claim 12 wherein the said polynucleotide comprises a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1 or SEQ ID No 2.

14. Use according to claim 12 or claim 13 wherein the said polynucleotide is under expression control of a plant operable promoter and further comprises a transcription termination region which is downstream of the protein encoding region of the said polynucleotide.

15. Use according to any one of claims 12 to 14 wherein the said promoter is selected from the group consisting of CaMV35S; FMV35S; NOS; OCS and E9.

16. Use according to claim 14 wherein the promoter is stomatal guard cell specific.

17. Use according to claim 16 wherein the promoter comprises the promoter active region of the sequence depicted as SEQ ID No 2.

18. Use of a polynucleotide according to any one of claims 12 to 17 in a method of producing plants with an increased number of stomata relative to control like plants wherein the said method is one according to any one of claims 1 to 9.

19. A polynucleotide which comprises a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1, but does not so hybridise when the said temperature is between 65 and 70°C.

20. A polynucleotide according to claim 19, comprising the sequence depicted in SEQ ID No. 2.

21. A polynucleotide according to claim 19 or claim 20, wherein the protein encoding region comprised by the polynucleotide is bounded by a plant operable promoter and terminator.

22. A plant transformation vector comprising the polynucleotide of any one of claims 19 to 21.

23. A plant transformation vector comprising a sequence which in the 5' to 3' direction comprises the complement of the polynucleotide according to any one of claims 19 to 21.

24. A plant transformation vector according to either of claims 22 or 23, wherein the protein encoding region of the polynucleotide is under expression control of a stomatal guard cell specific promoter.

25. The translational product encoded by the polynucleotide of any one of claims 19 to 21.

26. The product according to claim 25 having the activity of a fatty acid elongase.

27. Plant material which has been transformed with the polynucleotide according to any one of claims 19 to 21, the vector according to any one of claims 22 to 24, or a polynucleotide comprising a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1.

28. Plant material according to claim 27 which has been further transformed with a polynucleotide comprising a region encoding a protein capable of providing the plant material with resistance or tolerance to herbicides, insects, desiccation and/or fungal, bacterial or viral infections.

29. Plant material according to claim 28, wherein the protein capable of providing for herbicide resistance is selected from the group consisting of glyphosate oxido-reductase (GOX), 5-enol-pyruvyl-3-phosphoshikimate synthetase (EPSPS), phosphinothricin acetyl transferase (PAT), hydroxyphenyl pyruvate dioxygenase (HPPD), glutathione S transferase (GST), cytochrome P450, Acetyl-COA carboxylase (ACCase), Acetolactate synthase (ALS), protoporphyrinogen oxidase (PROTOX), dihydropteroate synthase, polyamine transport proteins, superoxide dismutase (SOD), bromoxynil nitrilase, phytoene desaturase (PDS), the product of the *tfd*A gene obtainable from *Alcaligenes eutrophus*, and known mutagenised or otherwise modified variants of the said proteins.

30. Plant material according to any one of claims 27 to 29, wherein the protein encoding sequences comprise 5' regions which encode: (i) a peptide which is capable of targeting the translation products of the regions to plastids such as chloroplasts, mitochondria, other organelles or plant cell walls; and/or (ii) non-translated translational enhancing sequences.

31. Plant material according to any one of claims 27 to 30, in which the polynucleotide used to transform the material is modified in that mRNA instability encoding motifs and/or fortuitous splice regions are removed, or plant preferred codons are used so that expression of the thus modified polynucleotide in a plant yields substantially similar protein having a substantially similar activity/function to that obtained by expression of the unmodified polynucleotide in the organism in which the protein encoding regions of the unmodified polynucleotide are endogenous, with the *proviso* that if - in respect of the herbicide resistance conferring regions - the thus modified polynucleotide comprises plant preferred codons, the degree of identity between the protein encoding regions within the modified polynucleotide and like protein encoding regions endogenously contained within the said plant and encoding substantially the same protein is less than about 70%.

32. A morphologically normal fertile whole plant regenerated from the material of any one of claims 27 to 31, the progeny of such plants, the seed of such plants and progeny, and parts of such plants and progeny.

33. A plant according to claim 32, selected from the group consisting of soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, grape, vines, cucumbers, peppers, citrus and nut plants.

34. A method of producing plants with an increased number of stomata relative to control plants comprising the steps of:

(i) inhibiting the function, or otherwise disrupting the activity, of an endogenous gene comprising a sequence which is complementary to one which when incubated at a temperature of between 60 and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS still hybridises with the sequence depicted in SEQ ID No. 1;

(ii) selecting the thus inhibited material;

(iii) regenerating the thus selected material into plants.

35. A method according to claim 34, wherein the plant material is transformed with the polynucleotide of any one of claims 19 to 21, or the vector of any one of claims 22 to 24.

36. A method according to either of claims 34 or 35, wherein the plants with an increased number of stomata are selected on the basis of a difference between non transformed control plants and the thus transformed plants when both are subjected post germination to at least one of the following: (i) elevated carbon dioxide concentrations, (ii) elevated calcium; (iii) extremes of temperature or pressure; (iv) reduced water availability; (v) elevated environmental pollutant gases, such as ozone, oxides of nitrogen or sulphur, and; (vi) elevated light conditions.

37. A method according to claim 36 wherein the said difference is selected from the group consisting of: (i) delayed flowering; (ii) altered growth characteristics; and (iii) an elevated stomatal index.

38. A method according to any one of claims 34 to 37, comprising the further steps of crossing the thus selected plants, or the progeny thereof, with non transformed like plants.

39. A method according to claim 38, wherein the plants eventually resulting from crosses are homozygous for the transgene.

40. Plants which result from the method of any one of claims 34 to 39, the progeny of such plants, the seed of such plants and progeny, and parts of such plants and progeny.

41. Parts of plants according to claim 40, selected from the group consisting of fruits, cut flowers and seeds.

42. Use of the polynucleotide of any one of claims 19 to 21, or the vector according to any one of claims 22 to 24, in method for the production of plants which have an increased number of stomata relative to non transformed control plants.

43. Expression regulatory sequences comprised by the sequence depicted in SEQ ID No 2.

44. An isolated polynucleotide comprising the sequence depicted as SEQ ID No 2.

45. An isolated polynucleotide comprising the sequence depicted as SEQ ID No 8.

46. Expression regulatory sequences comprised by the sequence depicted in SEQ ID No 8.

FIGURE 1

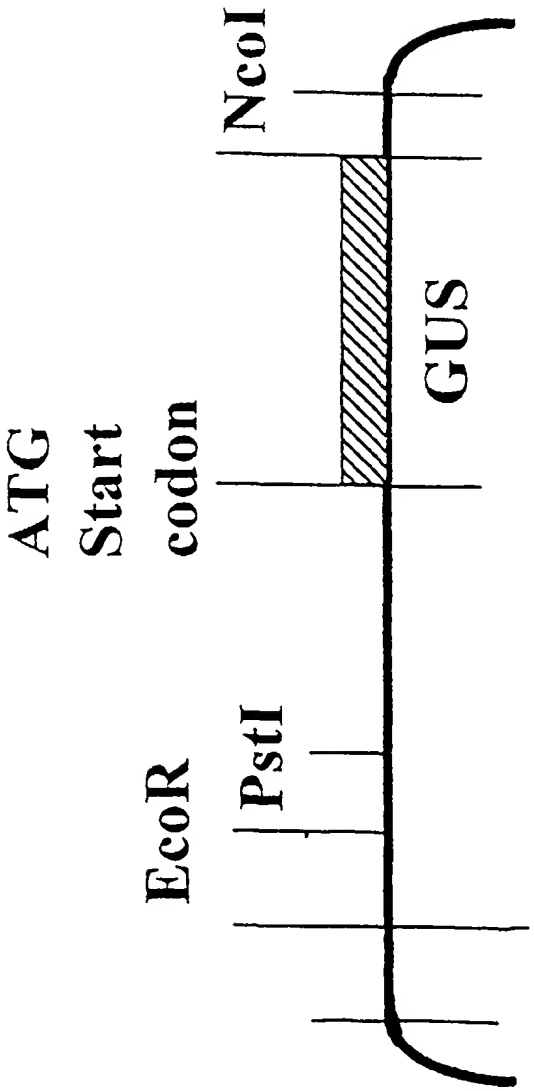


FIGURE 2

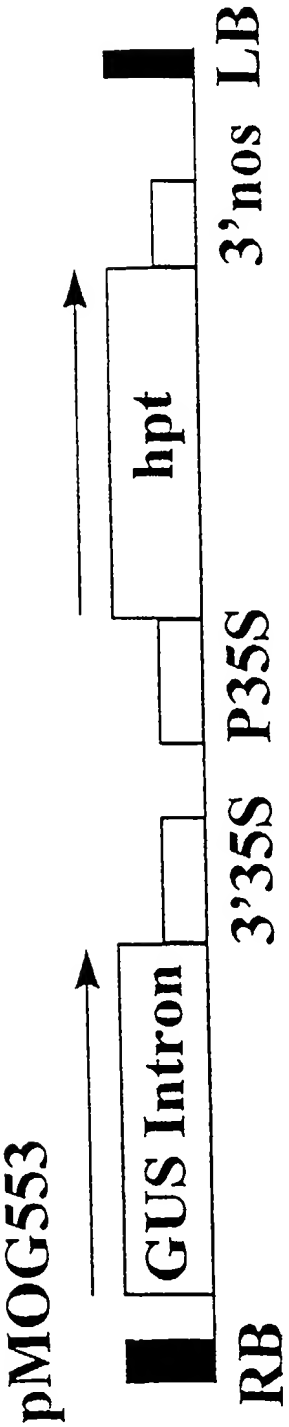


FIGURE 3

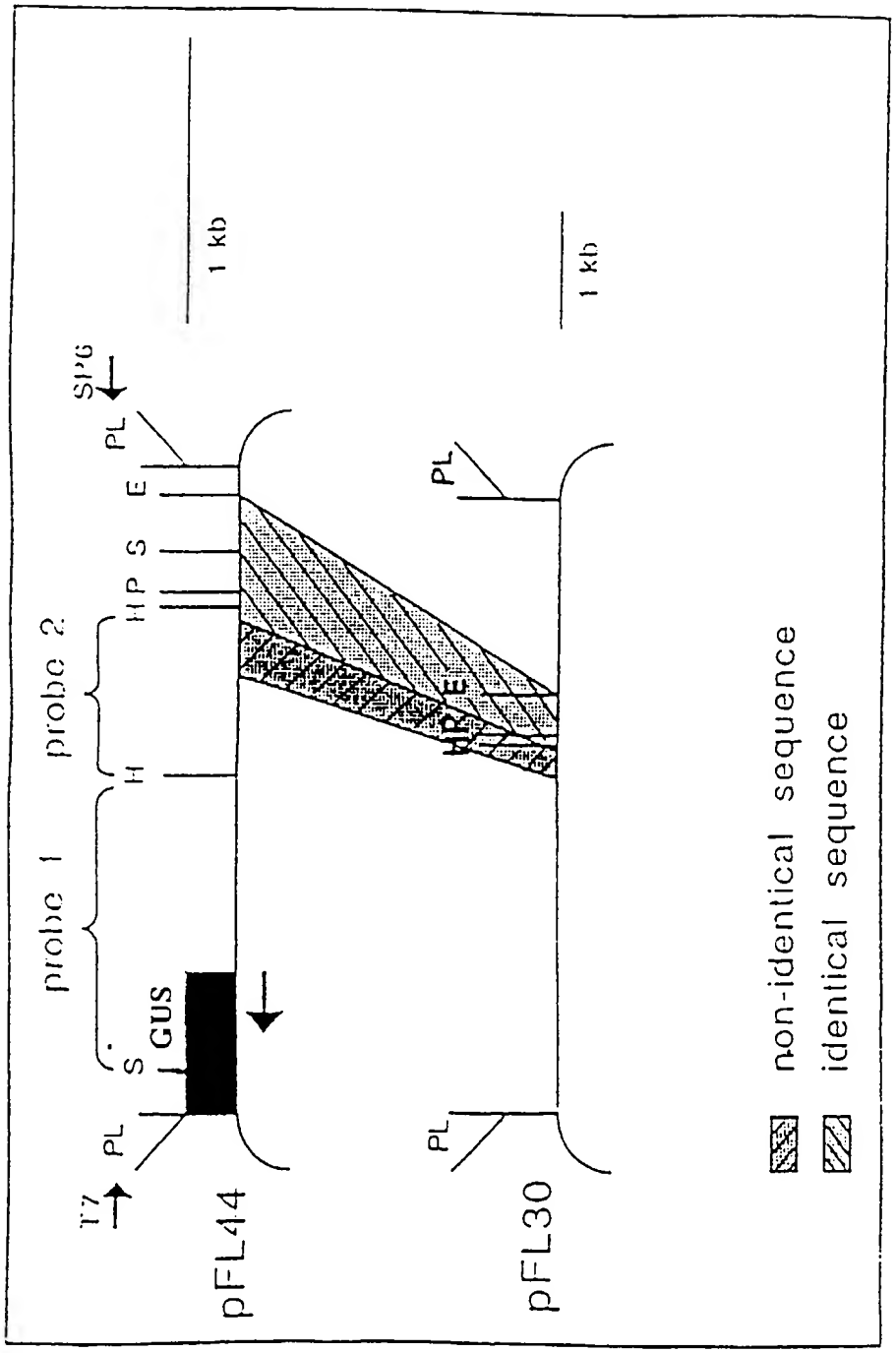
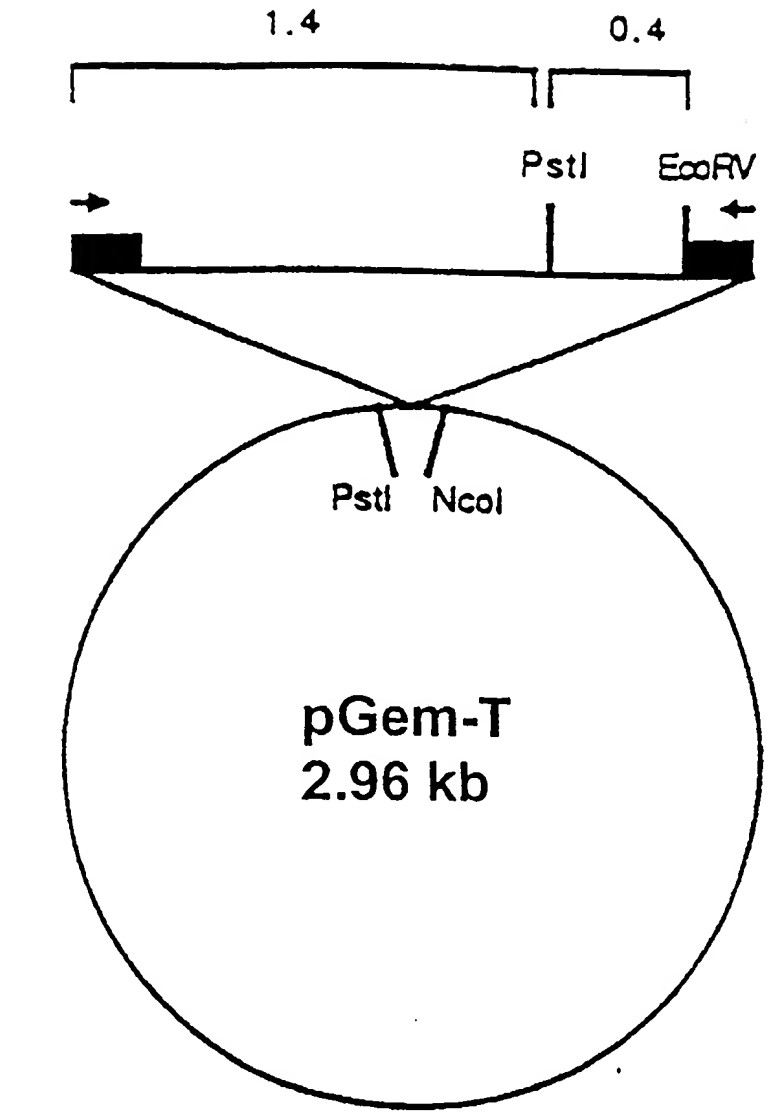
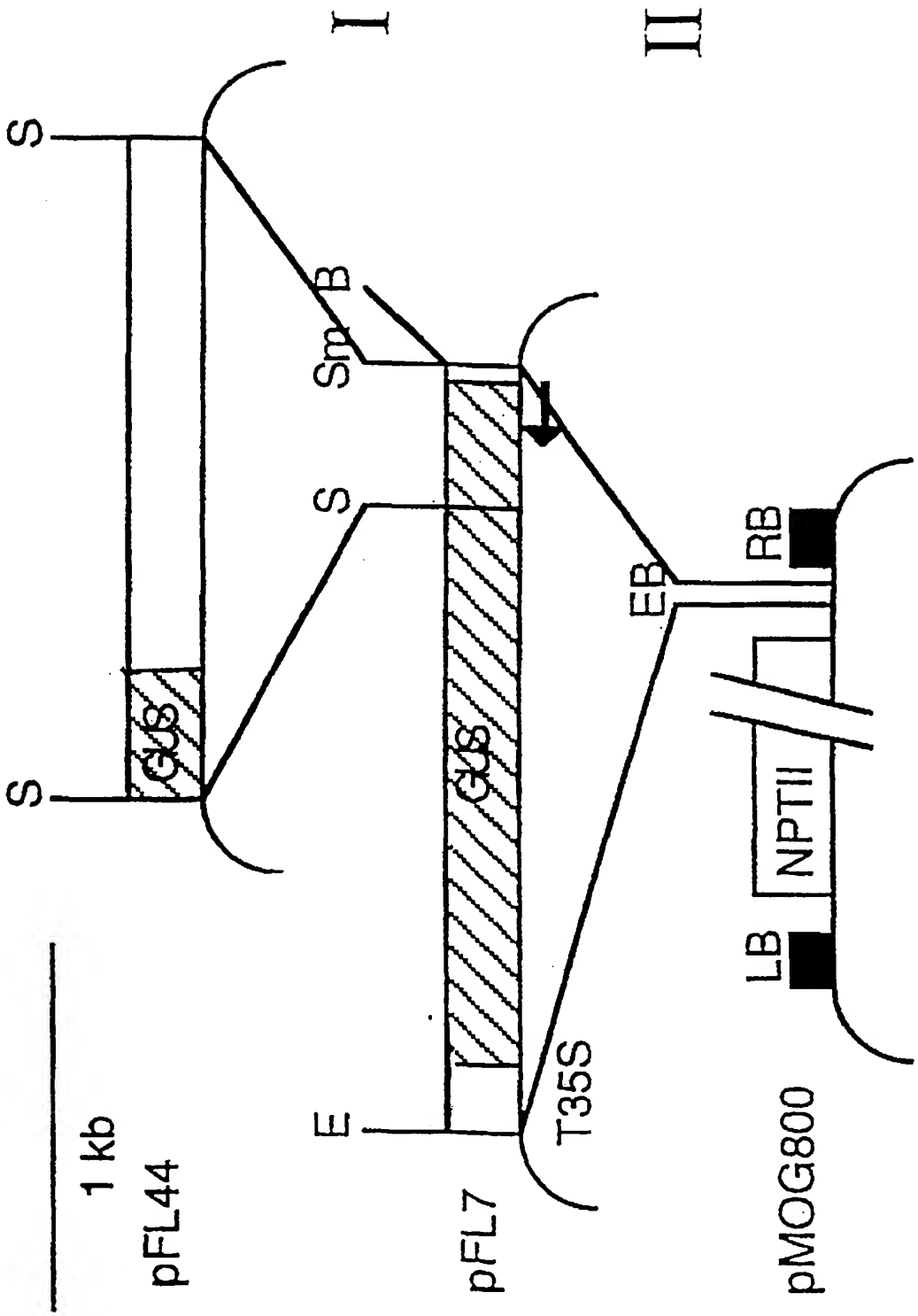

1 kb
1 kb
SP6
PL
E
S
H P
probe 2
H
probe 1
S
GUS
PL
T7
pFL44
PL
PL
pFL30
non-identical sequence
identical sequence

FIGURE 4



= Part of gus gene

FIGURE 5


I
II
S
S
GUS
Sm
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GUS
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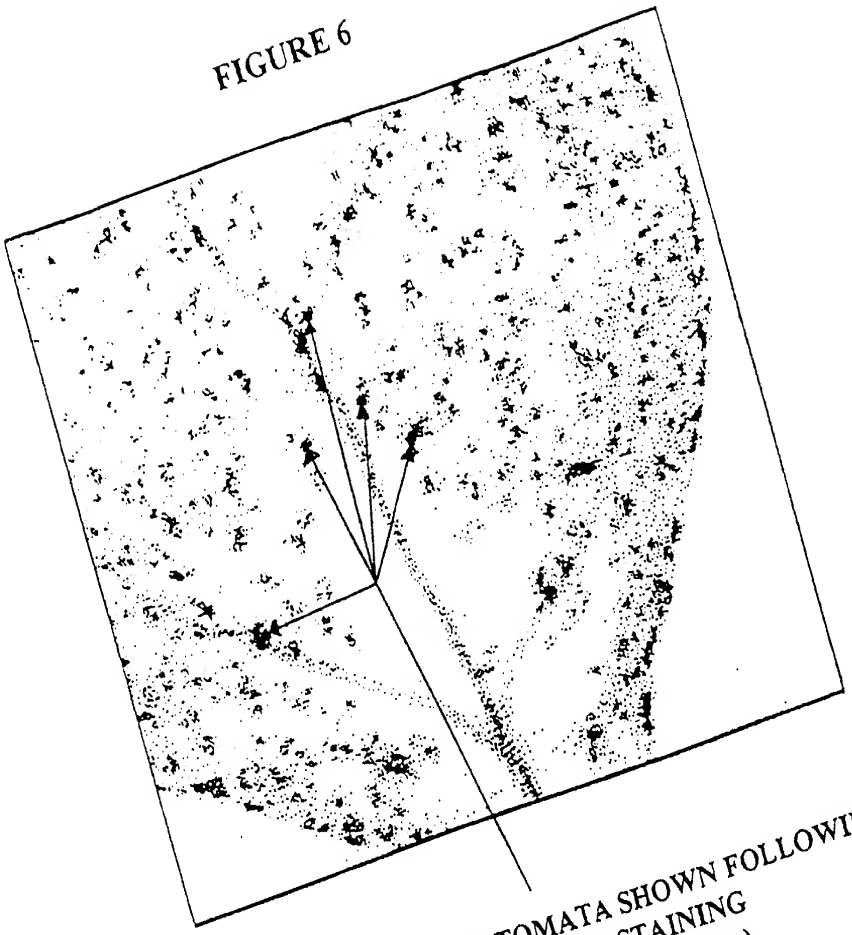
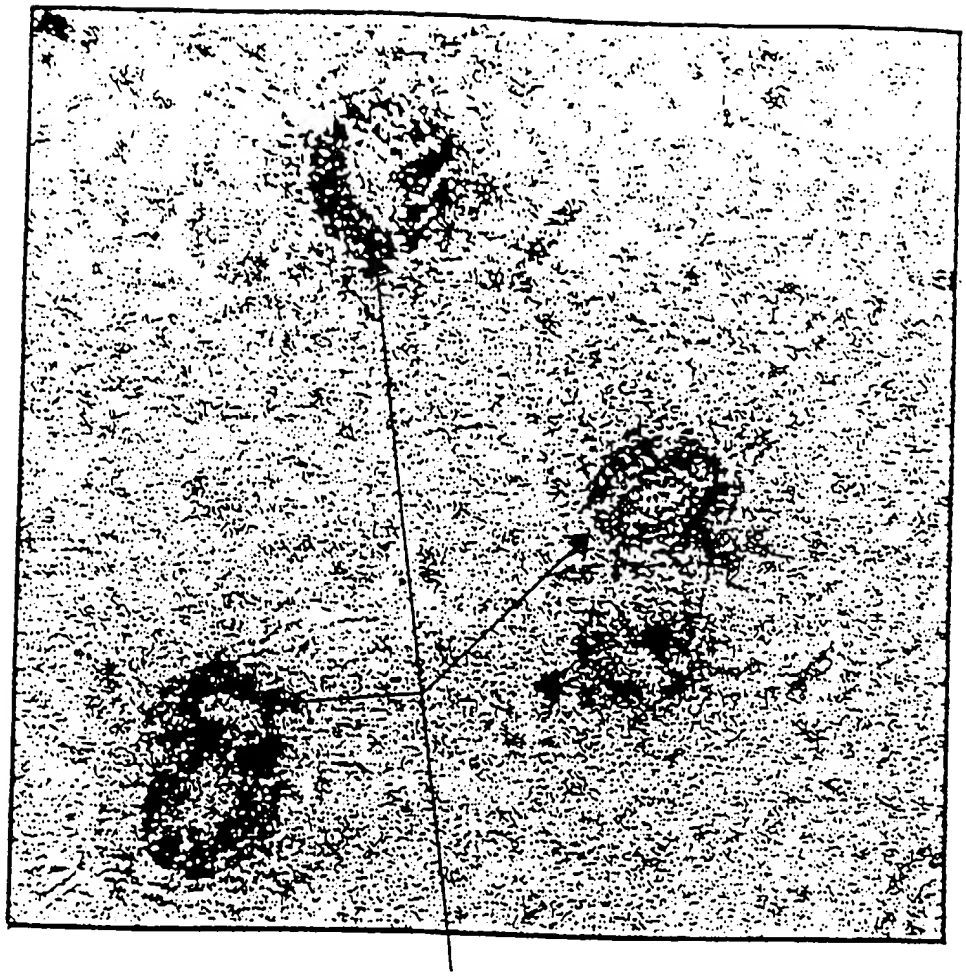
T35S
EB
RB
NPTII
LB
1 kb
pFL44
pFL7
pMOG800

FIGURE 6



ARABIDOPSIS LEAF STOMATA SHOWN FOLLOWING HISTOCHEMICAL STAINING
(Naturally blue colouration)

FIGURE 7



AT HIGHER MAGNIFICATION STAINING CAN CLEARLY BE SEEN IN THE GUARD CELLS OF THE STOMATA

SEQUENCE LISTING

<110> ZENECA LIMITED

<120> POLYNUCLEOTIDE SEQUENCES AND THEIR USE IN A METHOD OF PRODUCING PLANTS WITH AN INCREASED NUMBER OF STOMATA

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/01191

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/54 C12N15/82 C12N9/10 A01H5/00
A01H5/10 A01H5/08 A01H5/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 13582 A (DNA PLANT TECHN CORP) 9 May 1996 (1996-05-09) the whole document & JAMES DW ET AL: "directed tagging of the arabidopsis fatty acid elongation i (FAEI) gene with the maize transposon activator" PLANT CELL, vol. 7, March 1995 (1995-03), pages 309-319, cited in the application ---	19, 21-23, 25-33
X	WO 95 15387 A (CALGENE INC ;METZ JAMES GEORGE (US); LARDIZABAL KATHRYN DENNIS (US) 8 June 1995 (1995-06-08) figures 13,14 ---	19, 21-23, 25-33
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 July 1999	04/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bilang, J

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/01191

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	ROSCOE TJ ET AL.: "Brassica napus fatty acid elongase (FAE1) homolog mRNA" EMBL DATABASE, ACCESSION NUMBER U50771, 6 April 1996 (1996-04-06), XP002109729 the whole document	19, 21-23, 25-33
A	FERL RJ: "14-3-3-PROTEINS AND SIGNAL TRANSDUCTION" ANNUAL REVIEW IN PLANT PHYSIOLOGY AND PLANT MOLECULAR BIOLOGY, vol. 47, 1996, pages 49-73, XP002109730 page 61 - page 65; table 1	1
P,X	TODD JF ET AL.: "Arabidopsis thaliana fatty acid elongase 3-ketoacyl-CoA synthase 1 (KCS1) gene" EMBL DATABASE, ACCESSION NUMBER AF053345, 4 January 1999 (1999-01-04), XP002109731 the whole document & TODD J ET AL.: "KCS1 encodes a fatty acid elongase 3-ketoacyl-CoA stnthase affecting wax biosynthesis in Arabidopsis thaliana" THE PLANT JOURNAL, vol. 17, no. 2, 1999, pages 119-130,	19, 21-23, 25-33

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/01191

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9613582 A	09-05-1996	AU 703957 B	01-04-1999
		AU 3969995 A	23-05-1996
		CA 2203754 A	09-05-1996
		EP 0788542 A	13-08-1997
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		CA 2177598 A	08-06-1995
		EP 0731840 A	18-09-1996
		JP 9505739 T	10-06-1997